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Molecular identification of *Eimeria* spp. and *Eimeria bovis* in water buffaloes, Iraq

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Eimeria is a protozoan parasite of many wild and domestic animals including buffaloes resulting in a widespread disease known as coccidiosis that may impact production, well fare and health status. This study was designed to identify the prevalence of *Eimeria* spp. and *E. bovis* in buffaloes using conventional molecular technique. An overall 281 buffaloes of different sexes and ages of many rural areas in Wasit province were subjected during November (2022) to February (2023) to direct collect of fecal samples from the rectum. After molecular examination of all fecal samples by polymerase chain reaction (PCR), the results showed that 25.98 and 35.62% buffaloes were positive to *Eimeria* spp. and *E. bovis*, respectively. Furthermore, significant prevalence of *Eimeria* spp. being higher in < 1 year than older ones and Al-Kut than other study region, but not between females and males; while, *E. bovis* was more prevalent in < 1 year, Al-Kut and Al-Hay, and females. Additionally, values of odds ratio and relative risk were elevated significantly in < 1 year female buffaloes of Al-Kut region. From our point of view, this represents the first molecular study identifies *E. bovis* in buffaloes from Iraq. In conclusion, molecular based-PCR revealed a high efficacy in identification of *E. bovis*, and can be used in epidemiological investigations of coccidiosis in entire country. However, we suggest that the DNA sequence variation in ITS1 region of different *Eimeria* species should be conducted to view genetic mutations as well as genetic association with other worldwide strains. Also, extensive investigation is necessary to bridge the knowledge gap and providing for diagnosis this parasite in buffaloes as well as in other field animals.
Keywords: Coccidiosis, PCR, *Bubalus bubalis*, calf, wasit province. Iraq.

INTRODUCTION

Although coccidian parasites are host-specific, 12 of > 20 *Eimeria* species detected in cattle were found to infect buffaloes, and only 5 species can result in clinical signs including watery or bloody diarrhea, anorexia, weakness, dehydration and loss of weight leading to retardation of growth. However, only 2 species including *E. bovis* and *E. zuernii* have the greatest effect because of higher virulence and mortalities (Constable *et al*., 2016; Morgoglione *et al*., 2020; and Hastutiek *et al*., 2022). *Eimeria bovis* belongs to Eucoccidiorida Order of Apicomplexa Family, can enter by foods, water or surfaces that contaminate with infective (sporulated) oocysts. Inside cells of lamina properia and epithelium, schizogony undergo 2 asexual cycles to produce many small schizonts and to multiplying of these schizonts, respectively; and then one sexual cycle. This multiplication of oocysts causes destruction in mucosal cells to result in diarrhea in a host (Lassen *et al*., 2014; Golemansky, 2017; and López-Osorio *et al*., 2020).

Although morphology of the sporulated oocysts of different *Eimeria* species is followed as a gold-standard test, diagnosis of some species is very difficult due to morphological similarities between different species (De Waal, 2012; Alam *et al*., 2022). In last few decades, molecular characterization based on DNA amplification is applied to diagnosis different organisms including coccidial parasites in both human and animals, have approved the highly specific and sensitive findings to analysis the growth of parasite *in vitro* as well as in clinical materials (Chapman *et al*., 2013; Taha *et al*., 2021; and Ekawasti *et al*., 2022). Due to heterogeneity sequence base and length compositions, internal transcribed spacer 1 (ITS1) lends itself perfectly for rDNA transcription unit. Additionally, because it belongs to a family of multiple copy genes, ITS1 can provide a large number of potential PCR targets (Bass *et al*., 2015; Loo *et al*., 2022; and Ekawasti *et al*., 2023).

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In Iraq, a number of researchers have studied the prevalence rate of many species of *Eimeria* in different domestic and wild animals such as sheep (Kareem & Yücel, 2015; Al-Rubaie & Al-Saadoon, 2018; and Majeed *et al*., 2020), goats (Al-Amery & Hasso, 2002; Al-Bakray & Daoud, 2005), cattle (Hussin, 2016; and Aram, 2020), horses (Kalef, 2015), deer (Hadi *et al*., 2020; Kareem & Kawan, 2020), rabbits (Faraj, 2017), as well as in birds such as chicken (Al Se´adawy, 2013; El Iraqi & Melegy, 2014; and Ahmed & AlBakri, 2021), Quail birds (Mohammad, 2012; AL-Zarkoushi & AL-Zubaidi, 2022), geese and ducks (Al-Taee, 2022), and ostriches (Makawi & Jasim, 2020) using the traditional and molecular diagnostic assays. In Iraqi buffaloes, available online studies have been carried out traditionally to detect the parasite (Obayes *et al*., 2016; Sabbar & Al-Amery, 2020a; and AL-Lahaibi *et al*., 2021); while, the only molecular one was diagnosed the genus of *Eimeria* (Sabbar & Al-Amery, 2020b). Hence, this study was conducted to molecular identification of *E. bovis* in buffaloes for first time in Iraq, with estimating association between positive results and most important risk factors (age, sex, region and period).

MATERIALS AND METHODS

***Ethical approval*:** This study was approved and conducted under the license of the Scientific Committee of the College of Veterinary Medicine, University of Wasit (Wasit province) Iraq.

***Study animals and sampling*:** An overall 281 buffaloes of different sexes and ages of many rural areas in Wasit province were selected during November (2022) to February (2023) to collect fecal samples from the rectum into plastic labeled containers, cooled and transported using the plastic ice-bag to the laboratory.

***Molecular examination*:** Totally, 1 gm of each fecal sample was used for DNAs extraction following the manufacturer instructions (Geneaid, Korea). The concentration (ng/µl) and purity of extracted DNA samples were checked using the Nanodrop system at absorbance of A260 / A280 nm. Two sets of specific primers were used to detect *Eimeria* spp. [F: (5′-GCA AAA GTC GTA ACA CGG TTT CCG-3′) and R: (5′-CTG CAA TTC ACA ATG CGT ATC GC-3′)] and *E. bovis* [F: (5′-TCA TAA AAC ATC ACC TCC AA-3′) and R: (5′-ATA ATT GCG ATA AGG GAG ACA-3′)] based on the *ITS-1*. Following the manufacturer instructions of the Go Taq® Green Master Mix (M712) Kit, the Mastermix tubes of each set of primers were prepared at 25µl (5µl DNA Template, 2µl of each F and R primers, 16µl Nuclease-Free Water), and then subjected to PCR reaction using the Thermal Cycler-T100 (BioRad, USA) at the following conditions; 1 cycle for initial denaturation (95°C / 5 min); 35 cycles for denaturation (95°C / 30 sec), annealing (58°C / 30 sec) and extension (72°C / 2 min), and 1 cycle for final extension (72°C / 5 min). Conventional PCR assay using 1.5% agarose-gel stained with Ethidium bromed was carried out by electrophoresis at 100 Volt, 80 Am for 1 hour and the DNA fragments were visualized under the UV-Transilluminator (Clinx Science, China). The PCR products were considered positives for *Eimeria* spp. and *E. bovis* at approximately product sizes of 546 bp and 238 bp, respectively.

***Statistical analysis*:** The obtained results were examined statistically by the *t-test* and Odds Ratio in GraphPad Prism (GraphPad Software Inc., USA) to detect significant differences between molecular results and to estimate relationship between positive PCR results and epidemiological risk factors (age, sex, region and period) at $P<0.05$ (Gharban, 2023).

RESULTS

Application of conventional PCR assay revealed that 25.98% (73/281) and 35.62% (26/73) buffaloes were positive to *Eimeria* spp. and *E. bovis*, respectively (Figures 1, 2), at a product size of 546 bp and 238 bp, respectively (Figs. 3, 4).

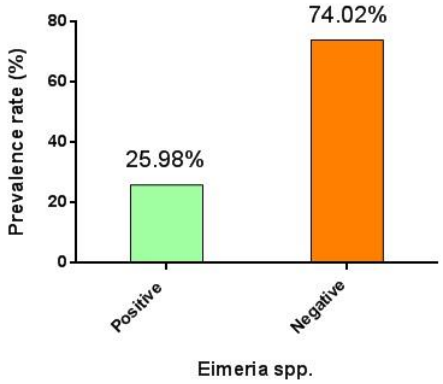

Figure 1. Total results for testing 281 fecal samples by conventional PCR assay to detect *Eimeria* spp.

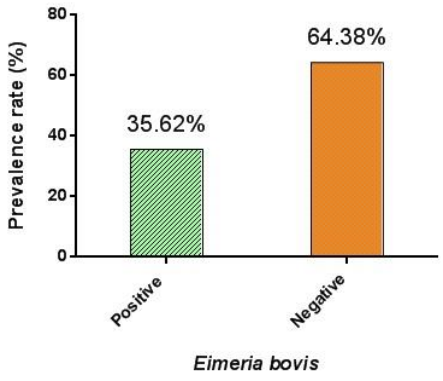

Figure 2. Total results for 73 positive fecal samples by conventional PCR assay to detect *Eimeria bovis*.

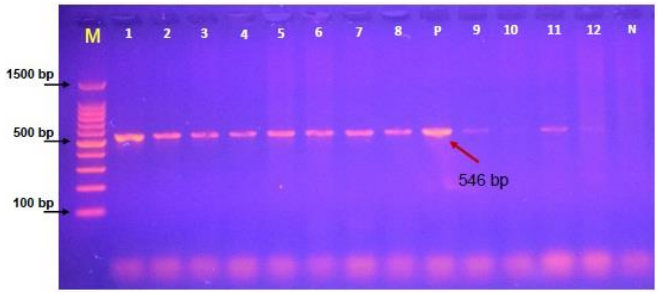

Figure 3. Agarose gel-electrophoresis of some PCR products at 100 Volt, 80 Am for 1 hour for detection *Eimeria* spp.

M: Ladder marker (1500-100 bp)
Lanes 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, and 13: Positive PCR products at a product size of 546 bp
Lane P: Positive control
Lane N: Negative Control

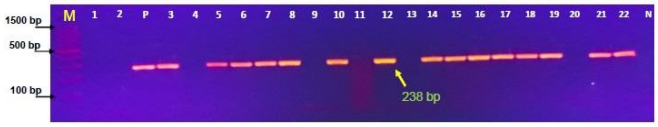

Figure 4. Agarose gel-electrophoresis of some PCR products at 100 Volt, 80 Am for 1 hour to detection *Eimeria bovis*

M: Ladder marker (1500-100 bp)
Lanes 3, 5, 6, 7, 8, 10, 12, 14, 15, 16, 17, 18, 19, 21, and 22: Positive PCR products at a product size of 328 bp
Lanes 1, 2, 4, 9, 11, 13, and 20: Negative PCR products
Lane P: Positive control
Lane N: Negative Control

Furthermore, analyses to detect association between positive results and some risk factors recorded a significant difference (P<0.05) in their values (Table 1). Regarding positive results to *Eimeria* spp., buffaloes of <1 year old were showed a significant elevation in prevalence of the parasite (58.46%) as well as in values of odds ratio (7.29) and relative risk (3.774), while significant reduction (P<0.05) was seen in old animals aged >7 years (9.52%, 0.259 and 0.329, respectively when compared to other age groups; >1-4 years (16.13%, 0.43 and 0.521, respectively) and >4-7 years (19.75%, 0.531 and 0.695, respectively).

Among study regions, prevalence rate of *Eimeria* spp. as well as values of odds ratio and relative risk were increased significant (P<0.05) in Al-Kut (48.94%, 3.522 and 2.285, respectively) and reduced significantly in Al-Suwaira (10.64%, 0.29 and 0.364, respectively) in comparison with the values of other regions; Al-Aziziyah (23.4%, 0.799 and 0.883, respectively), Al-Numaniya (15.22%, 0.458 and 0.541, respectively), Al-Hay (40.43%, 2.391 and 1.749, respectively) and Shaykh Sa'd (17.02%, 0.532 and 0.612, respectively).

For sex factor, although insignificant difference (P>0.05) was reported between females and males in values of prevalence rate (26.46% and 24.14%, respectively) and Odds ratio (0.978 and 1.022, respectively), females were appeared at a significant higher risk (P<0.03) for Eimeria's infection (1.1) than males (0.909).

Table 1. Association of positive results to *Eimeria* spp. with risk factors (Total No. 281)

Factor / Groups	Total No.	Positive No. (%)	Odds ratio	Relative risk
Age (Year)				
< 1	65	38 (58.46%) *	7.290*	3.774*
>1-4	93	15 (16.13%)	0.430	0.521
>4-7	81	16 (19.75%)	0.531	0.695
> 7	42	4 (9.52%)	0.259	0.329
p-value		0.0375	0.045	0.014
Region				
Al-Suwaira	47	5 (10.64%)	0.290	0.364
Al-Aziziyah	47	11 (23.4%)	0.799	0.883
Al-Numaniya	46	7 (15.22%)	0.458	0.541
Al-Kut	47	23 (48.94%)*	3.522*	2.285*
Al-Hay	47	19 (40.43%)	2.391	1.749
Shaykh Sa'd	47	8 (17.02%)	0.532	0.612
p-value		0.0105	0.045	0.018
Sex				
Female	223	59 (26.46%)	0.978	1.100*
Male	58	14 (24.14%)	1.022	0.909
p-value		0.0696	0.590	0.030

Significance * (P<0.05)

Concerning positive results to *E. bovis*, significant differences (P<0.05) were detected between the groups of study epidemiological factors (Table 2). Concerning age, significant increases (P<0.05) in prevalence rate of infection, odds ratio and relative risk were observed in buffaloes of <1 years age old (27.69%, 10.08 and 7.289, respectively) when compared to those of other age groups; >1-4 (1.08%, 0.072 and 0.072, respectively), >4-7 (7.41%, 0.721 and 0.0667, respectively) and > 7 (2.38%, 0.209 and 0.205, respectively). The findings of positivity were elevated significantly (P<0.05) in buffaloes of Al-Kut (23.4%) and Al-Hay (17.02%); while, values of odds ratio and relative risk were significantly (P<0.05) increased in animals of Al-Kut (4.781 and 3.441, respectively) only. Among other study region, the findings of prevalence rate, odds ratio and relative risk were recorded in Al-Aziziyah (4.26%, 0.427 and 0.371, respectively), Al-Numaniya (6.52%, 0.714 and 0.602, respectively), and Shaykh Sa'd (4.26%, 0.427 and 0.377, respectively) but not in Al-Suwaira that revealed no positive values (P>0.05).

Significantly, females were showed an increasing (P<0.05) in values of positivity (10.76%), Odds ratio (3.361) and relative risk (3.176) when compared to males (3.45%, 0.298 and 0.324, respectively).

Table 2. Association of positive results to *E. bovis* with risk factors (Total No. 281)

Factor / Groups	Total No.	Positive No. (%)	Odds ratio	Relative risk
Age (Year)				
< 1	42	18 (27.69%)*	10.080*	7.289*
>1-4	67	1 (1.08%)	0.072	0.072
>4-7	118	6 (7.41%)	0.721	0.067
> 7	54	1 (2.38%)	0.209	0.205
p-value		0.0375	0.012	0.035
Region				
Al-Suwaira	47	0 (0%)	0.000	0.000
Al-Aziziyah	47	2 (4.26%)	0.427	0.371
Al-Numaniya	46	3 (6.52%)	0.714	0.602
Al-Kut	47	11 (23.4%)*	4.781*	3.441*
Al-Hay	47	8 (17.02%)*	2.662	2.048
Shaykh Sa'd	47	2 (4.26%)	0.427	0.377
p-value		0.0485	0.001	0.002
Sex				
Female	223	24 (10.76%)*	3.361*	3.176*
Male	58	2 (3.45%)	0.298	0.324
p-value		0.0434	0.004	0.003

Significance * (P<0.05)

DISCUSSION

Due to complete lack of techniques that combine efficacy, reliability, objectivity, ease to use, and the capability to differentiate between all species, the epidemiology of bovine coccidial parasites has not been extensively studied. In order to identify specie-specific method, the inter-specific DNA sequence diversity within the ITS1 region has been studied in a number of researches. These studies discovered that ITS1 regions are less conserved than *rRNA* genes. Additionally, it demonstrates variations in DNA length and sequence, simplifies the design of primers, and lowers the possibility of cross-reactions between distinct species (Lew *et al*., 2003; Haug *et al*., 2007; and Hamidinejat *et al*., 2010). In the current study, conventional PCR assay based on the ITS1 region was served for identification and confirmation of *Eimeria* spp. and *E. bovis* in buffaloes with revealing that the prevalence rates were 25.98% and 35.62%, respectively. Worldwide, the prevalence rate of *Eimeria* spp. was 16% (Obayes *et al*., 2016), 38% (Azhar *et al*., 2017), 66% (Sabbar & Al-Amery, 2020 a/ b) and 32% (AL-Lahaibi *et al*., 2021) in Iraq, 3.4% in Bangladesh (Mamun *et al*., 2011), 40% in Greece (Founta *et al*., 2018), 30% in Indonesia (Nurhidayah *et al*., 2019), 55.9% in Italy (Morgoglione *et al*., 2020); 100% in Malaysia (Sani & Chandrawathani, 1987), and 60.7% in Venezuela (Ramirez *et al*., 2013). For both *Eimeria* spp. and *E. bovis* infections respectively, there were 36.4% and 8.3% (Rebouças *et al*., 1990), 43.6% and 21.1% (Rebouças *et al*., 1994), and 100% and 33-100% (Bastianetto *et al*., 2007) in Brazil; 12% and 33.33% (El-Sherif *et al*., 2000) and 28% and 10.8% (El-Alfy *et al*., 2019) in Egypt; 57.8% and 34.8% (Navjot *et al*., 2018) in India; 100% and 76.8% (Bahrami & Alborzi, 2013) and 35.3% and 15.1% (Tavassoli *et al*., 2018) in Iran; 25.1% and 50.3% (Guarino *et al*., 1997) in Italy; 14.6% and 19.1% (Hayat *et al*., 1994) and 58.8% and 52.3% (Hussain *et al*., 2017) in Pakistan; 16.5% and 32% (Padilla & Romero, 2007) in Philippine; 95.3% and 34.8% (Sayin *et al*., 1968) and 75% and 44.9% (Nalbantoglu *et al*., 2008) in Turkey. This variation in prevalence rate of infection between our study and other studies might belong to differences in diagnostic methods used. Although classical approach of morphology is less expensive as no highly technical instruments and facility is required, the sensitivity of the test is compromised and there might be more false positive cases. The accurate diagnosis of *Eimeria* species based on PCR assays has important implication for disease control, selection of treatment strategies and identification of alternative therapeutic approaches (Morgoglione *et al*., 2020). Other reasons of variation include environmental factors such as geographic region and type of management; animal factors such as age, sex, and breed; and number of samples tested. Therefore, these factors should carefully be evaluated when comparing the prevalence data from different studies.

In this study, the higher rate of *Eimeria* spp. and *E. bovis* infections in younger buffaloes of < 1 year was in agreement with that demonstrated by several studies (Navjot *et al*., 2018; Sabbar & Al-Amery, 2020 a/ b; and AL-Lahaibi *et al*., 2021). This might be explained by the fact young animals are less exposed to *Eimeria* but being more sensitive to infection and have high case fatality rate (Constable *et al*., 2016; Karimzadeh *et al*., 2022). While in adult buffaloes, frequent infections are believed to increase acquired immunity that could be the main reason for decreasing the oocyst excretion. Our data showed that geographical district has an effect on the prevalence of *Eimeria* spp. and *E. bovis* since significant higher prevalence was seen in Al-Kut and Al-Hay regions. This could be attributed to variation in management and environmental conditions because coccidian immunity is not solid and large quantities of oocysts may cause continues re-infection and raise the level of environmental contamination. Association between infection and sex factor was found to be insignificant with *Eimeria* spp. and significant with *E. bovis*. Several studies have revealed conflicted results as a number of researchers have found a variation between females and males (El-Alfy *et al*., 2019; Navjot *et al*., 2018; and Sabbar & Al-Amery, 2020 a/b) but not in others (Singh *et al*., 2012; AL-Lahaibi *et al*., 2021). This disparity among the findings cannot be explained exactly; however, the possible reason for higher prevalence rate in females in our study might be either the better management of males than females keeping in view their economic importance or to the number of males subjected to sampling. Additionally, alteration in the physiological condition of the animals during pregnancy and lactation might play a role in increasing of positivity in

females. Lloyd (1983) reported higher level of prolactin and progesterone hormones make the individual females more susceptible to any infection.

***Conclusion*:** This study represents the first molecular study identifies *E. bovis* in buffaloes from Iraq. We concluded that molecular based-PCR revealed a high efficacy in identification of *E. bovis*, and can be used in epidemiological investigations of coccidiosis in entire country. However, we suggest that the DNA sequence variation in ITS1 region of different *Eimeria* species should be further conducted to view genetic mutations as well as genetic association with other worldwide strains. Also, extensive investigation is necessary to bridge the knowledge gap and providing for diagnosis this parasite in buffaloes as well as in other field animals.

***Competing interests*:** No

***Funding*: none.**

***Ethical statement*:** all procedure of experiment was conducted based in animal walfare rules and under regulation of ethical comite of university.

***Availability of data and material*:** Raw data will be available when publisher requests.

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***Authors' contribution*:** MMA: Collection of fecal samples; OAA: Extraction of DNAs and preparation of Mastermix tubes; GJKA: PCR analysis (Gel-electrophoresis) and statistical analysis of obtained results. All authors have written and approved the final copy of the manuscript.

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